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Variation in the Hepatitis C Virus NS5a Region in Relation to Hypervariable Region 1 Heterogeneity During Interferon Treatment

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The putative interferon sensitivity determining region (ISDR) in the NS5a region of the hepatitis C virus (HCV) was analyzed in 13 interferon alpha (IFN-α) treated patients representing genotypes 1a, 1b, and 2b. These patients had previously been followed longitudinally during treatment with respect to viral load and to virus heterogeneity using the hypervariable region 1 (HVR1) sequence as a marker. In the present study, the NS5a region was analyzed for nonresponders and sustained responders using direct DNA sequencing. While the previous results of analyzing viral composition and load showed evidence of selection, no corresponding selection of specific NS5a ISDR sequences was observed in the nonresponders, and identical ISDR sequences were observed among both sustained responders and nonresponders. Thus, we cannot verify a correlation between ISDR sequence and the observed selection of IFN-α-resistant quasispecies demonstrated as a restriction of HVR1 heterogeneity. This indicates that the potential for using ISDR as a diagnostic or prognostic marker during IFN-α treatment is limited. ***J. Med. Virol. 56:33–38, 1998.***

KEY WORDS: interferon sensitivity; direct sequencing; quasispecies heterogeneity

INTRODUCTION

Treatment with IFN is currently the only approved therapy for patients with chronic hepatitis C virus (HCV) infection, although with limited and variable success. Unsuccessful IFN-α treatment has been associated with high heterogeneity of the virus population, high viral load, and genotype 1b in particular [Okada et al., 1992; Lau et al., 1993].

In a previous study of Swedish patients, we demonstrated a restriction of the virus population heterogeneity and the selection of specific quasispecies during IFN-α treatment by using direct sequencing of the hypervariable region 1 (HVR1) [Yun et al., 1996]. This study indicated that the mechanism behind IFN-α resistance is in part encoded virally. Similar observations have been reported previously, although using other approaches such as single-strand conformation polymorphism (SSCP) [Enomoto et al., 1994]. By comparative full-length sequencing of IFN-resistant and IFN-sensitive genotype 1b isolates of Japanese origin [Enomoto et al., 1995], a small sequence within the NS5a region of the HCV genome (codon 2209–2248) was implicated in IFN resistance. In a retrospective study of 84 Japanese patients infected with genotype 1b, Enomoto et al. [1996] further demonstrated a significant correlation between the number of amino acid mutations of this region, denoted interferon sensitivity determining region (ISDR), and the response to IFN treatment. Complete response did not occur in any of the patients infected with a quasispecies whose ISDR amino acid sequence was identical to the prototype 1b (hcv-j). In the defined intermediate-type group (those having one to three substitutions within the ISDR), 13% of the patients had a complete response to IFN treatment. In the defined mutant-type group (those having four or more substitutions within the ISDR compared to the prototype), all patients had a complete response. The strong correlation between ISDR amino acid sequence and response did indicate that the ISDR sequence had an important predictive value concerning the outcome of IFN-treatment in the individual patient. Also, by using in vitro and in vivo methods [Gale et al., 1997], NS5a was found to interact with the intracellular IFN-induced RNA-activated protein kinase (PKR), which is part of the cellular IFN-induced anti-

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viral defense. The interaction repressed the function of PKR, thus providing a plausible explanation for how a particular NS5a amino acid sequence might confer IFN resistance. It was shown that NS5a from both 1a and 1b prototype had the ability to bind and inactivate PKR, suggesting that a correlation between NS5a sequence and IFN resistance for 1a subtype isolates could exist, despite the fact that it has not yet been observed. It should thus be relevant to compare the ISDR amino acid sequence obtained from individual patient isolates not only to prototype 1b but also to prototype 1a. The findings of Enomoto et al. [1996] have been confirmed by others [Chayama et al., 1997] for Japanese isolates of genotype 1b. However, the correlation has been difficult to confirm for European and North American isolates [Hofgärtner et al., 1997; Khorsi et al., 1997; Squadrito et al., 1997; Zeuzem et al., 1997] of both 1a and 1b genotype, thereby questioning the relevance of ISDR as a potential diagnostic marker in IFN therapy. The issue remains open to debate, and it appears that the mechanism behind the virally encoded IFN resistance is not as simple as first indicated.

We extended our previous study [Yun et al., 1996] to sequence the NS5a region of the sequential patient isolates that had been characterized with respect to different virological parameters and dynamic changes within the HVR1 region during treatment. The HVR1 is a rapidly evolving and highly polymorphic region in the HCV genome with very few functional or structural constraints. It is therefore useful as a marker to study the heterogeneity and dynamic changes of the population of quasispecies in individual patient isolates, as a response to positive or negative selection pressure on other HCV regions. The aim of the present study was to investigate whether a relation between ISDR sequence and IFN resistance could be established in these Swedish patients, correlating to the observed selection of IFN-resistant quasispecies demonstrated previously.

MATERIALS AND METHODS

Thirteen patients with chronic hepatitis C were studied and confirmed by liver histology, persistently elevated alanine amino transferase (ALT) levels, anti-HCV antibody (2nd ELISA: Abott, Abott Park, IL; 2nd RIBA: Chiron, Emeryville, CA), and reverse transcription-polymerase chain reaction (RT-PCR) using primers from the 5′-noncoding region [Yun et al., 1993]. The patients were given IFN-α2b (3×10^6 units three times a week for 15 months) and subsequently divided into two groups on the basis of their treatment response [Yun et al., 1996]. Five of the patients included in the present study had normal serum ALT levels and were PCR-negative at the end of treatment and at a follow-up after 6 months, and were called sustained responders (SR). Eight of the patients included either did not respond to treatment or responded initially but experienced relapses. These patients had elevated serum ALT levels (>0.7 μkat/L) and PCR-positivity at the end of treatment or during the follow-up. The patients in this group were called nonresponders (NR) or nonsustained responders (NSR).

RNA was extracted from the frozen (−70°C) serum samples used in the previous study [Yun et al., 1996] (when still available), and samples collected at an additional time point 12 weeks into the IFN treatment. A 458 bp biotinylated PCR fragment (nt 6861–7319, D90208) was generated by seminested RT-PCR. RT and outer PCR were carried out in a single tube using 5 pmol each of primers ODJA21 (5′-CARYTICCITGYGAGCCCGAACCG-3′, annealing at nt 6837–6861) and ODJA22 (5′-AGRTGGIARIGGRCAICCRTGIACCAC-3′, annealing at nt 7319–7293) (where I = inosine; Y and R = IUPAC ambiguity codes for degenerate positions). The temperature profile was 42°C for 60 min, 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final soak at 72°C for 6 min. The outer PCR product was diluted 50–100-fold. Inner PCR was carried out with 10 pmol each of primers ODJA22 and ODJA23 (5′-biotin-GAYGTIRCIGTGYTIACKTCCATGCTCAC-3′, annealing at nt 6861–6889), with a temperature profile of 94°C for 5 min, followed by 30 cycles of 95°C for 20 sec, 60°C for 40 sec, 72°C for 2 min, and a final soak at 72°C for 10 min.

Direct solid-phase sequencing was carried out on both strands as previously described [Odeberg et al., 1995] using Cy5-labeled sequencing primers ODJA25 (5′-Cy5-CCAYATIACAGCAGIGRCGGC-3′) and ODJA26 (5′-Cy5-SIAKCGGITCRAAIGAGTCCA-3′), annealing at positions 6898–6919 and 7140–7120, respectively. The sequencing reactions were loaded onto an ALF Express automatic fluorescent sequencer (Pharmacia Biotech, Uppsala, Sweden) and analyzed as previously described [Odeberg et al., 1995]. Amplification, direct sequencing of the HVR1, and polymorphism analysis of these patients have been described previously [Yun et al., 1996]. The heterogeneity of HVR1 was defined by the number of polymorphic amino acid sites, deduced from the polymorphic nucleic acid sequence. Quantification of HCV was undertaken by competitive PCR [Yun et al., 1994]. In brief, HCV cDNA was coamplified with serially diluted cloned competitor, which consisted of the amplified 5′-noncoding region of HCV cDNA with an internal region substituted by a 21 bp lac operator sequence. The PCR product was specifically quantitated with a colorimetric assay. The sensitivity of the assay is approximately 10 genome equivalents, and the dynamic range is 10^1–10^7 genome equivalents/ml serum. Genotyping was done by the method of Okamoto et al. [1993] with minor modifications.

RESULTS

Part of the NS5a sequence was analyzed by direct DNA sequencing during IFN-therapy in 13 Swedish patients who, based on a previous study of longitudinal changes in viral load and heterogeneity of the HVR1 region [Yun et al., 1996], had shown evidence for selection of IFN-resistant quasispecies. The NS5a fragment sequences obtained are presented in Figure 1, showing

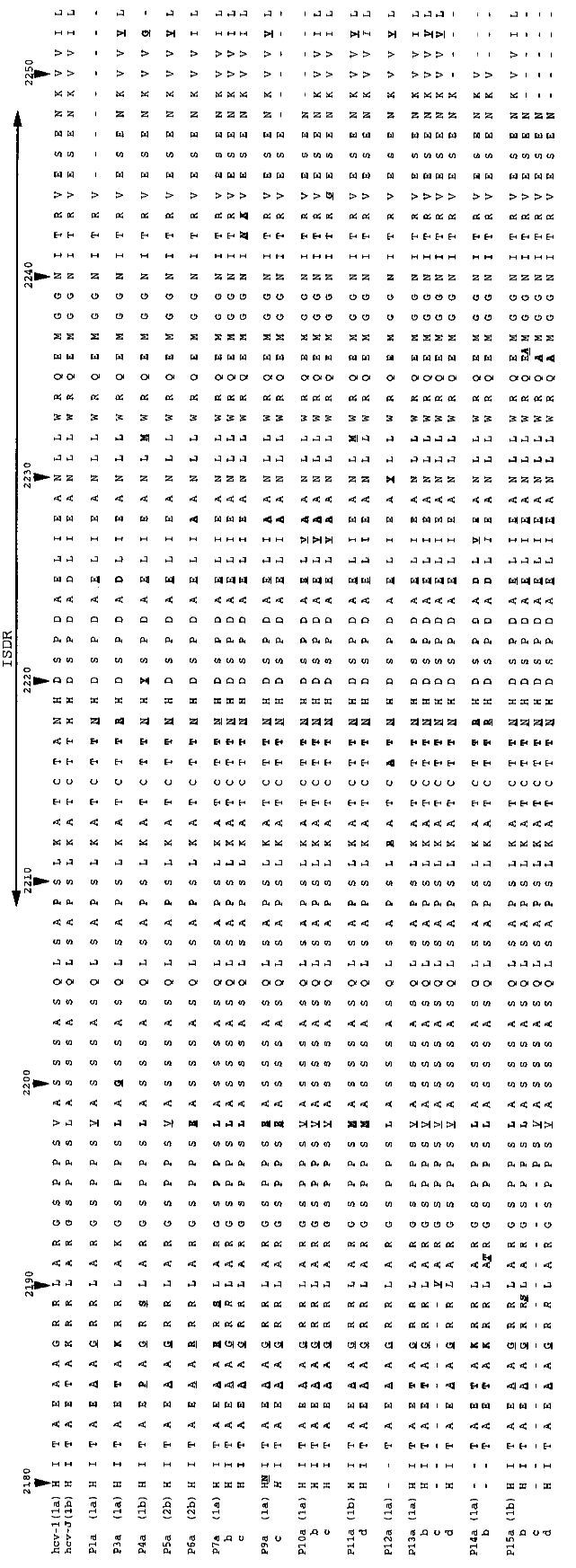

Fig. 1. Amino acid sequence of a part of the NS5a region, including ISDR, of patient isolates, together with prototype sequences of genotype 1a (accession number M62321) and 1b (D90208), where "a" denotes isolate before start of IFN treatment; "b," 12 weeks into IFN treatment; "c," end of treatment (15 months); and "d," follow-up six months after end of treatment. Boldface letters indicate amino acid substitutions compared to prototype 1a; underlined letters indicate substitutions compared to prototype 1b.

the amino acid sequence of the virus isolates. The putative ISDR is marked with an arrow. For some time points, however, NS5a sequence data could not be obtained because the sample was no longer available. The isolates were of genotype 1a, 1b, and 2b (Table I), with the majority being of genotype 1a. Table I also displays previous data [Yun et al., 1996] demonstrating a selection of quasispecies in the nonresponding patients and nonsustained responders, as determined by a reduction in viral load and polymorphic amino acid sites in the hypervariable region during IFN-α treatment.

It is noteworthy that none of the sequenced isolates had an amino acid sequence identical to the prototype sequences 1a or 1b. ISDR is regarded to be a conserved region, and the prototype sequence of 1a and 1 b differs only at three positions (1a versus 1b): codon 2217 Ala versus Thr, codon 2218 Asp versus His, and codon 2225 Glu versus Asp. The consensus sequence emerging among the Swedish patient isolates is in this respect a mixture of genotype 1a and 1b. Codon 2217 is threonine, codon 2218 is asparagine, and codon 2225 is glutamate. The ISDR of the individual patient isolates were compared with both 1a and 1b prototype sequences in terms of amino acid substitutions, since the NS5a region of both prototypes has been demonstrated to be involved in the inhibition of the IFN-induced RNA-activated protein kinase [Gale et al., 1997].

The deviations between prototype and isolate sequences, measured as the number of amino acid substitutions in the ISDR region compared with prototypes for genotype 1a and 1b, are depicted in Table I. No correlation could be found between the IFN-α response and the number of amino acid substitutions (see Table I) versus either prototype when comparing patients with a sustained response to NR and NSR patients. There was no correlation between the previously observed selection of IFN-resistant specific quasispecies and any specific NS5a ISDR sequence (Fig. 1).

For seven out of the eight NSR and NR patients included in this study, NS5a sequence data exist from more than one time point during treatment. Five of the seven patients (P7, P10, P11, P14, P15) exhibited amino acid mutations within ISDR over the time interval studied (Fig. 1). For one of the remaining two (P13), amino acid mutations occurred just outside ISDR N-terminal, and in the other (P9), an amino acid heterogeneity was eliminated. In fact, all but one of the patients showed changes in amino acid composition in the regions flanking ISDR, and several of these amino acid changes were nonconservative. The amino acid positions involved differed among the isolates, and only two amino acid positions were involved in longitudinal

TABLE I. Summary of Obtained Data

Sample[a]	Histology	Genotype	Response to IFN	HCV load[b]	Polymorphic amino acid sites (HVR1)[c]	Amino acid substitution within ISDR vs. Prototype	
						1a	1b
P1a	CAH	1a	SR	2×10^5	4	1	2
P3a	CAH	1a	SR	3.2×10^3	0	3	1
P4a	CPH	1b	SR	2.5×10^5	2	3	4
P5a	CAH/Ci	2b	SR	5.1×10^6	5	1	2
P6a	CAH/eCi	2b	SR	3×10^5	0	2	3
P7							
a	CAH	1a	NSR	8×10^6	6	1	2
b				n.a.	n.a.	1	2
c				6.4×10^3	0	1	2
d				8×10^6	7	3	4
P9							
a	CAH/eCi	1a	NSR	5×10^5	8	2	3
b				n.a.	n.a.	n.a.	n.a.
c				1.3×10^5	0	2	3
d				2×10^5	0	n.a.	n.a.
P10							
a	CPH	1a	NR	2.8×10^6	2	3	4
b				n.a.	n.a.	3	4
c				5×10^5	0	4	5
d				2×10^5	4	n.a.	n.a.
P11							
a	CAH	1b	NR	8×10^4	3	2	3
b				n.a.	n.a.	n.a.	n.a.
c				8×10^3	1	n.a.	n.a.
d				1.3×10^6	1	1	2
P12							
a	CAH	1a	NR	2×10^5	1	4	5
b				n.a.	n.a.	n.a.	n.a.
c				5.6×10^5	0	n.a.	n.a.
d				7×10^5	0	n.a.	n.a.
P13							
a	CAH	1a	NR	3.1×10^6	5	1	2
b				n.a.	n.a.	1	2
c				7.1×10^5	3	1	2
d				2.5×10^6	8	1	2
P14							
a	CAH	1a	NR	2.2×10^6	5	4	2
b				n.a.	n.a.	3	1
c							
d				2.6×10^6	4	n.a.	n.a.
P15							
a	CAH	1b	NR	2.5×10^6	2	1	2
b				n.a.	n.a.	2 (ambig)	3 (ambig)
c				1.3×10^6	0	2	3
d				5×10^6	4	2	3

[a]a = isolate before start of IFN treatment; b = 12 weeks into IFN treatment; C = end of treatment (15 months); d = follow-up six months after end of treatment. CAH = chronic active hepatitis; CPH = chronic persistent hepatitis; Ci = cirrhosis; eCi = early cirrhosis; SR = sustained responder; NR = nonresponder; NSR = nonsustained responder; n.a. = not available; ambig = ambiguity; a polymorphic amino acid position. The five first columns represent data from Yun et al. [1996].
[b]HCV load as genome equivalent/ml serum.
[c]Number of polymorphic amino acid positions within HVR1 as determined by direct sequencing, used as a measure of quasispecies heterogeneity.

changes in more than one patient: codon 2189 (P7 and P15) and codon 2252 (P11 and P13).

In the previous study [Yun et al., 1996], patient P12 showed the most obvious example of selection for an IFN-resistant strain, with elimination of the limited viral heterogeneity and increase of viral load throughout treatment. The pre-IFN treatment sequence available displays three unusual amino acid substitutions in positions strongly conserved in the other isolates and in the prototype sequences—codon 2212 (K→R), codon 2216 (T→A), and codon 2230 (N→Y)—and it would fall into the mutant-type group when compared with either prototype, having four or more amino acid substitutions. A phylogenetic analysis of part of the NS5a region (including ISDR) was carried out to see if a correlation between the clustering based on amino acid sequence and the response to treatment could be found. The result is shown in Figure 2. No apparent relation on amino acid level is seen among the NR and NSR isolates or among the SR isolates.

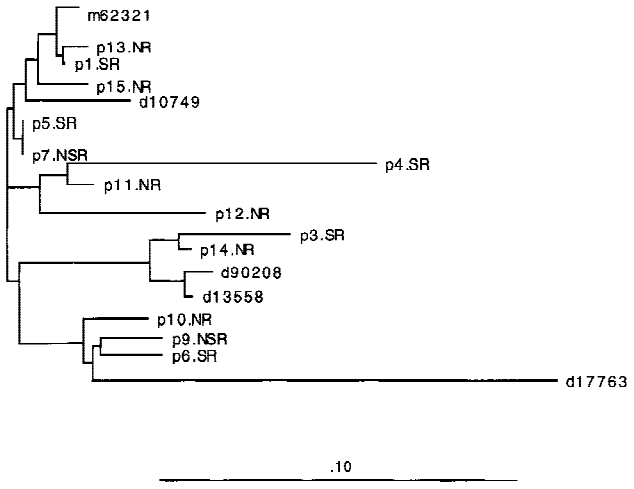

Fig. 2. Phylogenetic tree analysis based on the NS5a amino acid sequence, codon 2178–2259, of the different patient isolates. The tree was constructed with the NEIGHBOR program in the PHYLIP software package (version 3.51c), kindly provided by J. Felsenstein, using the amino acid distance matrix obtained by using the Dayhoff PAM matrix. SR denotes sustained responder; NSR, nonsustained responder; and NR, nonresponder. m62321 = hcv-1; d90208 = hcv-j; d10749 = HC-J1; d13558 = HC-J4; d17763 = NLZ1.

DISCUSSION

To our knowledge, this is the first study in which a comparison of HVR1 variability with NS5a sequences during IFN treatment is made. The strength of the material is that selection of specific quasispecies during IFN treatment previously was established in the NR and NSR patients. Direct sequencing data from the NS5a region can thus be interpreted in view of the previous data for each isolate. From our material, no specific amino acid sequence pattern or number of substitutions within the NS5a ISDR that correlates with the IFN response can be found. For example, the ISDR amino acid sequence of the IFN-resistant isolate of patient P13 (NR) was identical before, during, and after IFN treatment (see Fig. 1) to the ISDR amino acid sequences of the IFN-sensitive isolates obtained before treatment of patients P1 and P5 (both SR). Also, the number of ISDR amino acid substitutions compared to the prototype sequences appears to be evenly scattered among SR and NR/NSR isolates in this study (see Fig. 1).

Regarding the longitudinal amino acid mutations observed in NR and NSR patients during IFN treatment, no specific codon position was targeted. It can be argued that the number of patients included in this study is too small to rule out any correlation. However, the data from the previous study [Yun et al., 1996] concerning viral load and viral heterogeneity based on longitudinal sequence analysis of HVR1 clearly showed evidence for selection of IFN-resistant quasispecies among the NR and NSR patients, and if a conserved ISDR sequence was associated with IFN resistance, it should be discernible in these patients. The fact that no correlation to a specific amino acid sequence pattern or number of substitutions within the ISDR of these isolates could be established, as compared to the SR patients, indicates that the potential of the ISDR amino acid sequence as a marker in IFN treatment is limited, and would have been a poor predictor of IFN response in the patients of this study. This is further supported by the phylogenetic analysis of a larger region of NS5a that also did not show any relation on amino acid level within the two groups of responders, NSR + NR and SR, respectively.

The reason for the discrepancy compared with previous data [Enomoto et al., 1995, 1996] could be due to regional differences in genetic diversity [Khorsi et al., 1997]. The isolates in our study are all of Swedish origin, with the majority being genotype 1a. The original correlation between NS5a sequence and IFN response [Enomoto et al., 1995], as well as later reports supporting these findings [Enomoto et al., 1996; Chayama et al., 1997], has been based on Japanese isolates of genotype 1b. However, a study of 32 European isolates of genotype 1a and 1b could not identify a correlation between ISDR amino acid sequence and IFN treatment [Zeuzem et al., 1997], in agreement with our results for Swedish isolates. Two more studies [Khorsi et al., 1997; Squadrito et al., 1997] based on, respectively, 87 and 65 European isolates (French and Italian) of mainly 1b genotype could verify neither a correlation between pretreatment ISDR amino acid sequence nor response to IFN.

The relative amino acid conservation between isolates and between time points in our study, despite the marked changes in quasispecies heterogeneity as judged from the HVR1 sequences of the same isolates, suggests that NS5a encodes a protein with structural and/or functional constraints. The fact that changes in NS5a amino acid composition was observed over time in all seven NR and NSR, and in five cases occurring within the ISDR, is intriguing. It should be emphasized that these changes were observed with direct sequencing, thus providing a consensus view of the quasispecies population. In other words, changes in composition of the predominant quasispecies has occurred. This selection, however, does not necessarily have to be directed toward the NS5a region, but could also be just an indication of a positive selection pressure acting on a beneficial mutation somewhere else in the genome, as has previously been concluded from the HVR1 data.

In conclusion, we are unable to find a correlation between the NS5a ISDR sequence and the previously demonstrated selection of IFN resistant quasispecies in these patients. This indicates that the potential of NS5a ISDR sequence as a general prognostic tool in IFN treatment of individual patients is very limited.

REFERENCES

Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Suzuki Y, Murashima N, Ikeda K, Kumada H (1997): Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. Hepatology 25:745–749.

Enomoto N, Kurosaki M, Tanaka Y, Marumo F, Sato C (1994): Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment revealed by single-strand conformation polymorphism analysis. Journal of General Virolgy 75:1361–1369.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C (1995): Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b: Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. Journal of Clinical Investigation 96:224–230.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C (1996): Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. New England Journal of Medicine 334:77–81.

Gale M, Korth M, Tang N, Tan S-L, Hopkins D, Dever T, Polyak S, Gretch D, Katze M (1997): Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 230:217–227.

Hofgärtner WT, Polyak SJ, Sullivan DG, Carithers RL Jr, Gretch DR (1997): Mutations in the NS5A gene of hepatitis C virus in North American patients infected with HCV genotype 1a or 1b. Journal of Medical Virology 53:118–126.

Khorsi H, Castelain S, Wyseur A, Izopet J, Canva V, Rombout A, Capron D, Capron JP, Lunel F, Stuyver L, Duverlie G (1997): Mutations of hepatitis C virus 1b NS5A 2209–2248 amino acid sequence do not predict the response to recombinant interferon-alpha therapy in French patients. Journal of Hepatology 27:72–77.

Lau JY, Davis GL, Kniffen J, Qian KP, Urdea MS, Chan CS, Mizokami M, Neuwald PD, Wilber JC (1993): Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet 341:1501–1504.

Odeberg J, Yun Z, Sönnerborg A, Uhlen M, Lundeberg J (1995): Dynamic analysis of heterogeneous hepatitis C virus populations by direct solid-phase sequencing. Journal of Clinical Microbiology 33: 1870–1874.

Okada S, Akahane Y, Suzuki H, Okamoto H, Mishiro S (1992): The degree of variability in the amino terminal region of the E2/NS1 protein of hepatitis C virus correlates with responsiveness to interferon therapy in viremic patients. Hepatology 16:619–624.

Okamoto H, Tokita H, Sakamoto M, Horikita M, Kojima M, Iizuka H, Mishiro S (1993): Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. Journal of General Virology 74:2385–2390.

Squadrito G, Leone F, Sartori M, Nalpas B, Berthelot P, Raimondo G, Pol S, Brechot C (1997): Mutations in the nonstructural 5A region of hepatitis C virus and response of chronic hepatitis C to interferon alpha. Gastroenterology 113:567–572.

Yun ZB, Lindh G, Weiland O, Johansson B, Sönnerborg A (1993): Detection of hepatitis C virus (HCV) RNA by PCR related to HCV antibodies in serum and liver histology in Swedish blood donors. Journal of Medical Virology 39:57–61.

Yun Z, Lundeberg J, Johansson B, Hedrum A, Weiland O, Uhlen M, Sönnerborg A (1994): Colorimetric detection of competitive PCR products for quantification of hepatitis C viremia. Journal of Virology 47:1–13.

Yun ZB, Odeberg J, Lundeberg J, Weiland O, Uhlen M, Sönnerborg A (1996): Restriction of hepatitis C virus heterogeneity during prolonged interferon-alpha therapy in relation to changes in virus load. Journal of Infectious Diseases 173:992–996.

Zeuzem S, Lee JH, Roth WK (1997): Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon alpha. Hepatology 25:740–744.